Attempts to Create Rickets in Mice Using a Calcium Deficient Diet

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Abstract: Previous research with chickens produced rickets by reducing the calcium content of the diet. When rickets occurred, RNA was extracted from the growth plates for microarray analysis. The results showed a difference in expression of more than 80 RNAs between the normal and rachitic growth plates. Because many more antibodies to mouse cellular proteins have been developed than to chicken proteins, the present research was an attempt to produce rickets in mice using a low calcium diet. In the first experiment, mice that were just weaned were fed 0.2, 0.4, or 0.6 % calcium. The average starting weight was 11.8 grams. After three weeks, the average weights were 26.6, 24.9, and 23.5 grams for the respective treatments. Microscopic examination of the growth plates failed to reveal any effect of treatment. A second experiment used dietary treatments of 0.1% and 0.6% calcium. Average starting weight of the 20 mice was 13.5 grams. After three weeks, the weights of the respective groups were 27.2 and 27.6 grams. Upon dissection, the tibias of the 0.1% calcium group seemed more fragile; however, the bone ash of the 0.1% calcium group was 44.1% and that of the 0.6% calcium group was 39.5%. A microscopic examination of the growth plates of tibia from the 0.1% group suggested less organized cellular arrangement and more cellular netting than was present in the 0.6% calcium group. Immunohistochemistry was used to identify four proteins in the growth plates of the tibias. The proteins, which had been identified in the chicken research, were eukaryotic translation elongation factor 1 delta, activating transcription factor 7, DNA primase p49 subunit, and adaptor related protein complex 1 gamma 1 subunit. The immunohistochemistry was completed, but no difference in expression of the proteins was found between growth plates from mice fed 0.1% calcium and those fed 0.6% calcium.

Introduction

The idea for this research project stemmed from a previous project with chickens fed calcium deficient diets to produce rickets. When rickets occurred, RNA was extracted from the growth plates for microarray analysis. The results showed a difference in expression of more than 80 RNAs between the normal and rachitic growth plates (3). Because many more antibodies to mouse cellular proteins have been developed than to chicken proteins, the present research was an attempt to produce rickets in mice using a low calcium diet.

Calcium deficient diets in young individuals may result in the disorder called rickets. When insufficient calcium is absorbed by the bone, the bones do not harden properly and are too soft to support the weight of the body (7). A bone is made up of three layers; the zone of

proliferation where the cells actively divide, the zone of pre-hypertrophy where the cells get larger and become more round, and the zone of hypertrophy where the cells undergo apoptosis, or programmed cell death. Once apoptosis occurs in the zone of hypertrophy blood vessels invade the zone to remove cellular fragments. After the cells are removed mineralization of the bone occurs. In calcium deficient individuals, such as those with rickets, the blood vessels in the zone of hypertrophy appear blunted.

Calcium is also an essential nutrient not only used to build strong bones and teeth, but also in muscle contraction, blood clotting, nerve impulse transmission, regulating heartbeat, and fluid balance within cells. It is an important activator of oxidative metabolism as well. The calcium in the cytosol of a cell is lower in concentration than in the extracellular spaces. The large concentration gradient is maintained by the active transport of calcium across the plasma membrane and endoplasmic reticulum by Ca ATPase. Ca ATPase actively pumps two calcium ions out of the cytosol by using two ATP molecules while transporting in two or three protons (5).

Calcium is involved with the protein phosphorylase kinase, which is a protein with four subunits. The gamma subunit can be blocked, inhibiting activity. Full activity of the gamma subunit requires calcium bound to another subunit, which is called calmodulin. Even low calcium concentrations can activate phosphorylase kinase by inducing conformational change in calmodulin that causes it to bind and expel the gamma subunit's inhibitor. The activation of phosphorylase kinase increases the rate of glycogen breakdown which is linked to the rate of muscle contraction. This is important because glycogen breakdown provides fuel for glycolysis to generate the ATP needed for muscle contraction. In fact, calcium also regulates the citric acid cycle, or Krebs Cycle, at several points to directly affect ATP production. It activates pyruvate

dehydrogenase complex which produces acetyl CoA, as well as both isocitrate dehydrogenase and alpha ketogluterate dehydrogenase (5).

Calcium can also regulate protein synthesis like it does muscle contraction and ATP production through RNA polymerase. RNA polymerase catalyzes the synthesis of a complementary strand of RNA from a DNA template. The three types of RNA polymerases that calcium is involved in are; polymerase I, which transcribes DNA to synthesize rRNA; polymerase II, which catalyzes the transcription of DNA to synthesize precursors of mRNA and most snRNA; and polymerase III, which transcribes DNA to synthesize ribosomal 5S rRNA, tRNA, and other small RNAs. If the gene is copied, RNA polymerase II finds the TATA box, which is a promoter to start the initiation of transcription. For the RNA polymerase to function, an assembly of transcription factors needs to take place, the transcription factors help center the polymerase on the TATA box. One of the transcription factors contains a TATA box binding protein which helps transcription for polymerase I, II, and III. Phosphorylation activates the polymerase II complex and elongation factors come into effect (6).

Phosphorylation can occur with calcium activating a protein kinase, which moves to the nucleus to activate a CREB protein (cAMP response element binding). The activated CREB protein binds to a cAMP response element (CRE), which activates associated genes (6).

Materials and Methods

Two experiments were performed on two different groups of mice, one with 0.2%, 0.4%, or 0.6% calcium, the other with 0.1% and 0.6% calcium in the diets (4). The diets were initially mixed with the lowest calcium concentration and adjusted with limestone. The composition of the 0.2% calcium diet is given in Tables 1, 2, and 3. The composition of the 0.1% calcium diet is given in Tables 4 and 5. The mice were weaned at 3 weeks of age and the mice were fed their

respective diets, beginning at weaning, for 3 weeks. Mice come into puberty at about seven weeks of age. This is a critical time of life for bone accretion because bone mineral density increases 40-50% (2). The mice were weighed each week to compare growth. At six weeks of age the mice were euthanized using carbon dioxide gas.

Samples of tibias from mice in all of the groups were collected to determine ash content and to produce slides for hematoxylin-eosin staining to detect cell growth and the development of rickets in the growth plates of the mice. Immunohistochemistry was also performed to detect four proteins in the growth plate of the tibias. The proteins, which had been identified in the chicken research, were eukaryotic translation elongation factor 1 delta, activating transcription factor 7, DNA primase p49 subunit, and adaptor related protein complex 1 gamma 1 subunit. The process of immunohistochemistry staining used antibodies that were purchased. Growth plates from the calcium deficient and normal mice were examined (1).

Table 1:

Corn grain	80.4% of diet
Soybean meal	16.4%
Dicalcium phosphate	0.68%
Salt	0.40%
Vitamin and trace mineral mix	1.00%
Methionine	0.10%
Soybean oil	1.00%

Formula for 0.2% calcium diet from first mouse experiment

Table 2:
Calculated Analysis:

Protein	14%
Calcium	0.2%
Total Phosphorus	0.5%

Table 3:
Vitamin and trace mineral mix:

Vitamin A	3000 IU
Vitamin D	500 IU
Vitamin E	10 mg
Vitamin K	0.5 mg
Thiamin	2.0 mg
Riboflavin	3.6 mg
Pantothenic acid	10 mg
Niacin	27 mg
Vitamin B12	0.01 mg
Choline	500 mg
Vitamin B6	3 mg
Biotin	0.1 mg
Folic acid	0.25 mg
Ethoxyquin	50 mg
Manganese	50 mg
Zinc	50 mg
Selenium	0.1 mg

Table 4:

Corn grain	80.66% of diet
Soybean meal	16.0%
Dicalcium Phospate	0.18%
Mono-Sodium Phosphate	0.61%
Salt	0.13%
Potassium Chloride	0.42%
Soybean Oil	1.00%
Vitamin and trace mineral	1.00%

Formula for 0.1% calcium diet from the second mouse experiment

Table 5:
Vitamin and trace mineral mix

Vitamin A	3000 IU
Vitamin D	500 IU
Vitamin E	10 mg
Vitamin K	1.0 mg
Thiamin	2.0 mg
Riboflavin	5.0 mg
Niacin	25 mg
Pantothenic acid	6.0 mg
Pyridoxine	3.0 mg

Biotin	0.1 mg
Folic acid	0.5 mg
Vitamin B12	0.02 mg
Choline	500 mg
Antioxid	25 mg
Selenium	0.1 mg

Results

The mice in the first experiment (0.2%, 0.4%, and 0.6% calcium groups) were weighed weekly. Their average starting weight was 11.8 grams. After three weeks, the average weights were 26.6, 24.9, and 23.5 grams for the respective treatments. The weights were relatively close and did not suggest the onset of rickets. Microscopic examination of the growth plates stained with hemotoxylin and eosin failed to reveal any effect of treatment. The growth plates all looked very similar with organized columns of cells in the zone of proliferation. The second experiment (0.1% and 0.6% calcium) showed similar results, the average starting weights for the mice were 13.5 grams. After three weeks, the weights of the respective groups were 27.2 and 27.6 grams. Upon dissection, the tibias of the 0.1% calcium group seemed more fragile; however, the bone ash of the 0.1% calcium group was 44.1% and that of the 0.6% calcium group was 39.5%. A microscopic examination of the growth plates of the tibias from the 0.1% group suggested less organized cellular arrangement and more cellular netting in the zone of proliferation than was present in the 0.6% calcium group.

Figure 1 shows a photograph of a growth plate of a normal mouse which received a diet sufficient in calcium (0.6%). Chondrocytes in the resting zone (R), zone of proliferation (PL), zone of prehypertrophy (PH), and hypertrophy (H) are visible. Blood vessels, however, are not easily distinguishable. The chondrocytes in a normal growth plate are very organized and arranged in columns, separated by a strongly stained matrix rich in proteoglycans (8). A few of

the mice fed the 0.1% calcium diet showed growth plates with disorganized chondrocyte arrangement and more netting than observed in mice fed the 0.2%, 0.4%, and 0.6% diets, suggesting a calcium deficiency (8).

Figure 2 is an example of the immunohistochemistry staining technique used on a normal mouse growth plate. The darker stained parts of the photograph are the reaction caused by the antibody and highlights the protein that was isolated in growth plate. The staining did not result in many easily readable slides, which demonstrates how difficult it was working with such small growth plates with small cells. In fact, no difference in expression of the proteins was found between growth plates from mice fed 0.1% and those fed 0.6% calcium (8).

Figure 3 is a photograph of a normal chick growth plate stained with hematoxylin-eosin. The growth plate is much larger and easier to read. The blood vessels are also seen clearly. Figure 4 shows a growth plate of a chick with rickets. The blood vessels in the plate are blunted along the border of chondrocytes and mineralized bone (3).

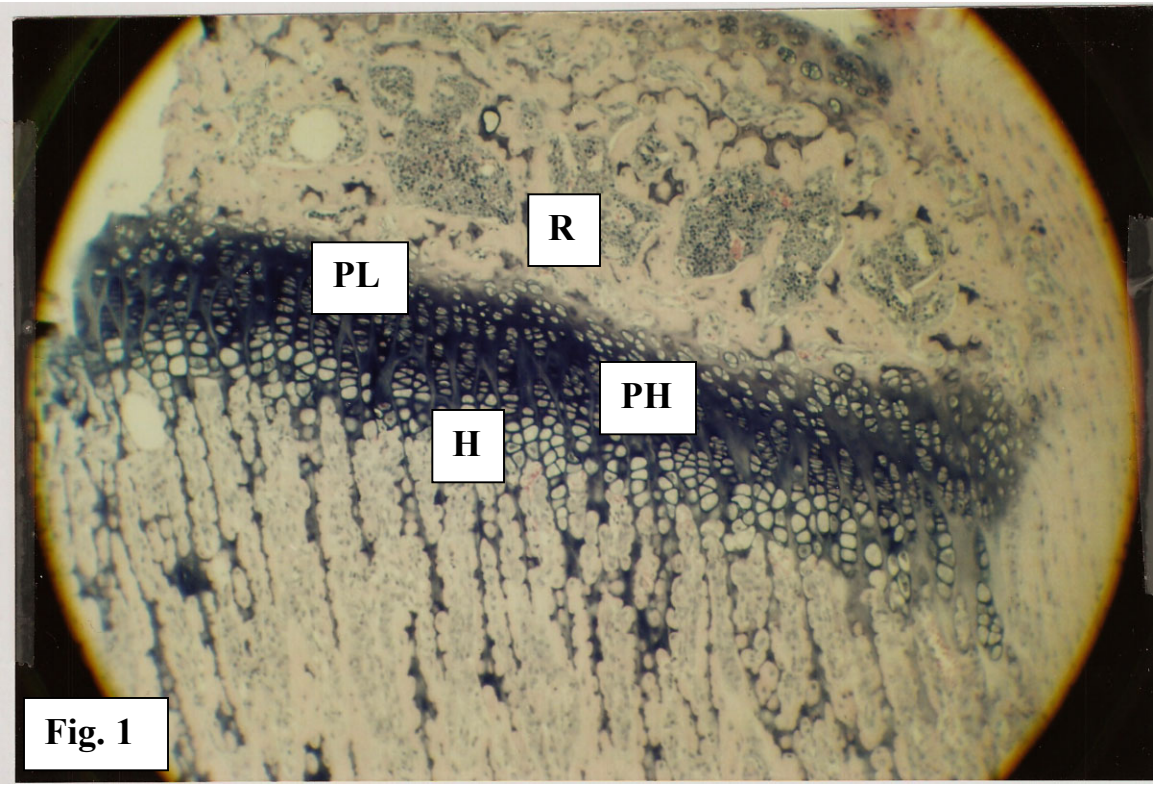

Figure 1 shows the growth plate of a mouse receiving 0.6% calcium in their diet or a normal amount of calcium for bone growth. R is the resting zone, PL is the zone of proliferation, PH is the zone of prehypertrophy, and H is the zone of hypertrophy. This section of growth plate was stained with hematoxylin-eosin.

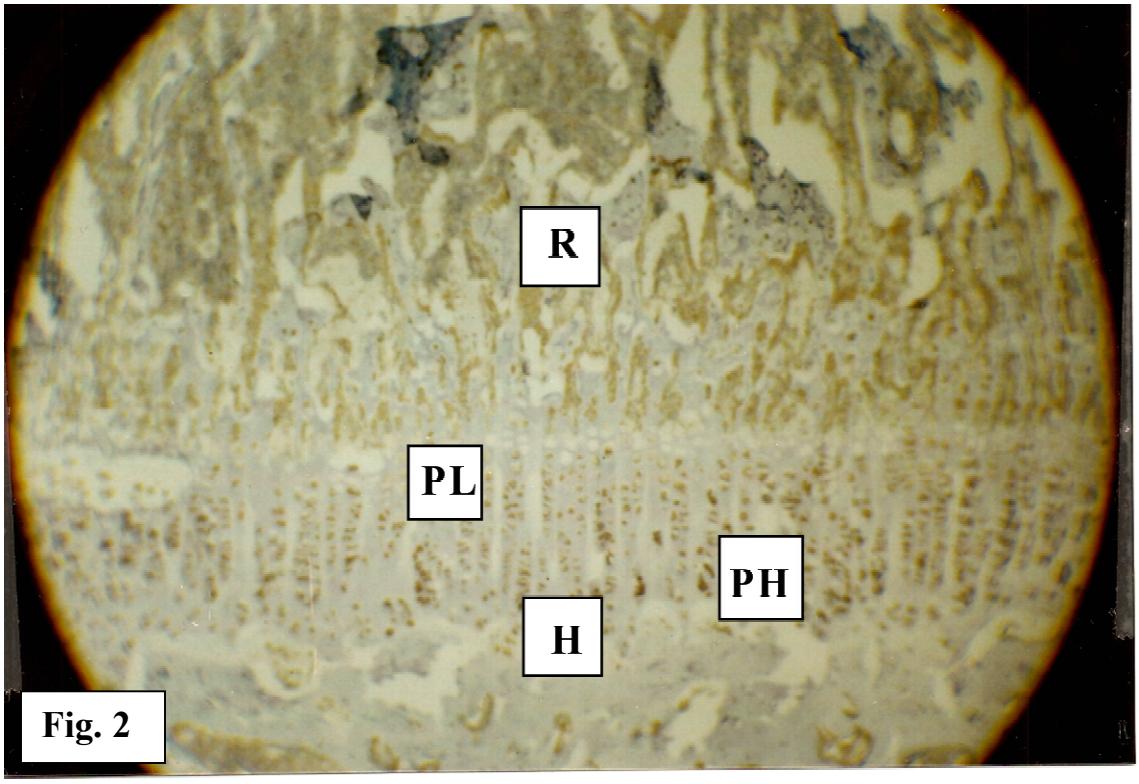

Figure 2 shows a growth plate from a mouse fed 0.6% calcium. This slide was stained with immunohistochemistry and the darker sections indicate the protein that was isolated.

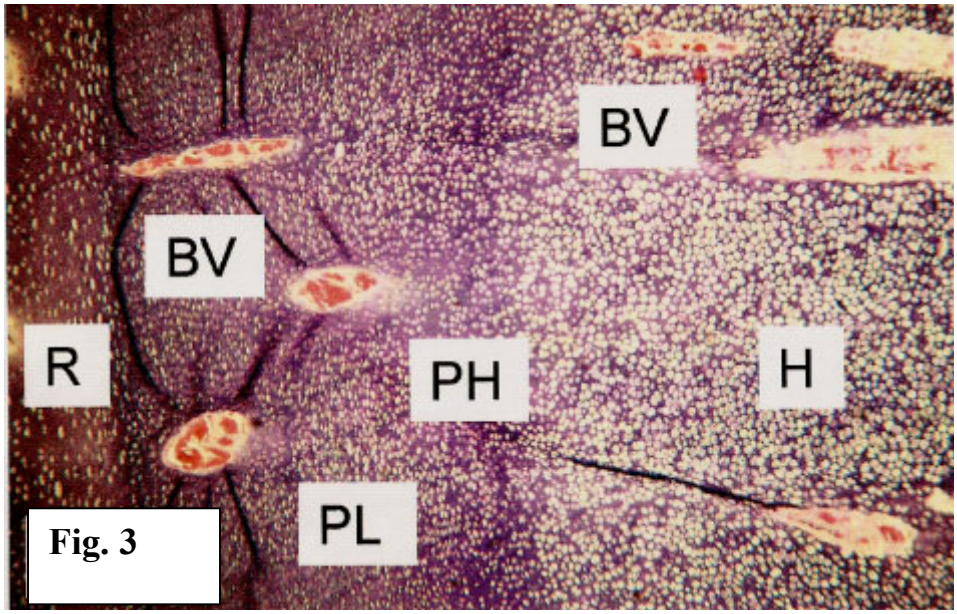

Figure 3 shows a normal growth plate of a chick. In this photograph BV or blood vessels are clearly and easily seen.

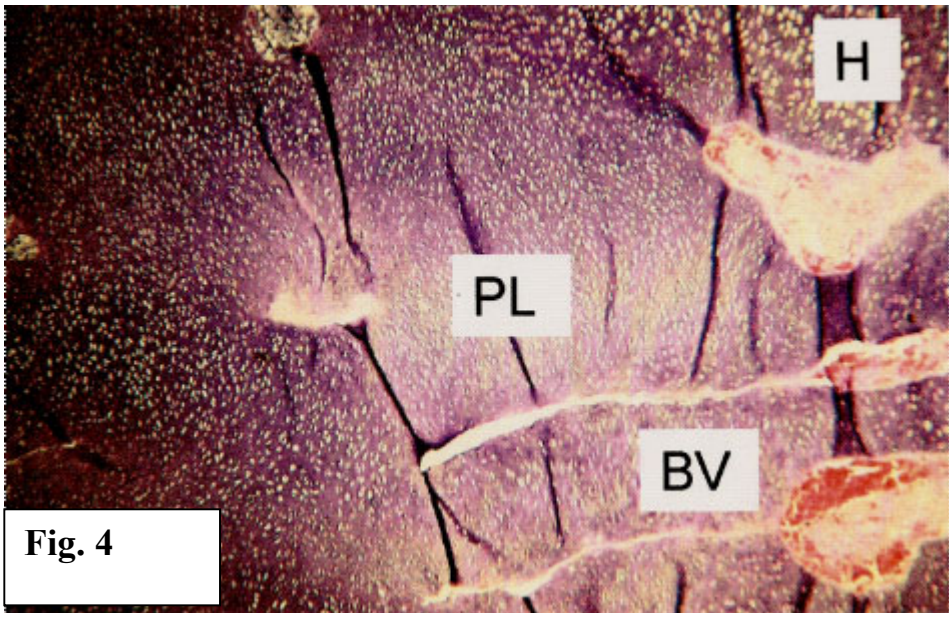

Figure 4 shows a growth plate of a chick with rickets and the BV in this case are large and blunt.

Discussion

Establishing a calcium deficiency in these two groups of mice was difficult and challenging. The first experiment yielded little results which lead to a second experiment with mice fed an even lower percentage of calcium. The weights of the mice were not affected by

their respective diets and the stained slides of the growth plates also showed little difference from group to group. The bone ash results collected also yielded little or no difference among the groups. A few of the bones from the 0.1% calcium group seemed fragile and weak resulting in some slides which showed disorganization of the chondrocytes in the growth plate and more of the proteoglycan netting suggesting a calcium deficiency or the development of rickets.

The comparison of the mouse growth plate to that of the chicken's demonstrates why it was so hard to work with mice in generating calcium deficiencies. The chicken growth plates are much larger and the cells are much easier to work with as well as easier to read and comprehend. The mice growth plates are small with small cells making it harder to read and even make slides.

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